

Study of vigor and esterase activity under NaCl stress deciphering overlap between gametophyte and sporophyte in annual *Medicago* species

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Abstract

To study the overlapping of gene expression between sporophyte and gametophyte phases, three annual species of the *Medicago* genus; *M. truncatula*, *M. ciliaris* and *M. aculeata* were used. First, we measured the germination rate of seeds and pollen grains as well as the length of young seedlings and the length of the pollen tubes under different levels of NaCl concentrations (0 mM, 68 mM, 102 mM and 136 mM). Afterwards, we measured the biochemical activity of plants based on electrophoresis of esterase enzymatic activity under salt stress treatments. The results showed that genotype *M.acl4* of *M. aculeata* species is the most tolerant in the two stages, while the genotypes *M.cil 1* and *M.trur1* are the most sensitive of *M. ciliaris* and *M. truncatula* species, respectively. The separation of the esterase activities by electrophoresis revealed four bands with quantitative and qualitative changes in relation to stress in the same sensitive genotype *M.cil1* of *M. ciliaris* species. The presence of a correlation between measured traits like vigor, as well as the connection between esterase profiles during periods of salt stress in both the sporophyte and gametophyte stages, may unveil a shared pattern of gene expression.

Keywords: *Medicago* - NaCl stress - Tolerance index - Sporophyte- Gametophyte -Esterase activity-Overlap.

Abbreviations: ANOVA_ Analysis of variance; TI_ Tolerance index; Est_ esterase; SD_ Standard deviation.

Introduction

The *Medicago* genus has presented adaptation, particularly in Algeria, constituting an extremely rich and diversified phylogenetic patrimony (Abdelguerfi et al., 1988). The annual species of this genus are omnipresent in semiarid regions, supporting the pastoral activity. All land plants like *Medicago* genus share a life cycle that alternates between sporophytes and gametophytes phases. Therefore, sporophytes and gametophytes are distinct and separate life cycle of organisms, varying in physiology, morphology, persistence, ecology, and usually ploidy, while sharing a common genome (Sigel et al., 2018). It has long been recognized that natural selection during the haploid gametophytic phase of the plant life cycle may have widespread importance for rates of evolution and the maintenance of genetic variation (Beaudry et al., 2020). Recent theoretical advances have further highlighted the significance of gametophytic selection for diverse evolutionary processes. Genomic approaches offer exciting opportunities to address key questions about the extent and effects of gametophytic selection on plant evolution and adaptation (Beaudry et al., 2020) to environmental stress. On the other hand, salinity (or changes in salinity) (Cui et al., 2018) is one of the most challenging abiotic stresses that threatens plant growth and development (Yao et al., 2018). However, the *Medicago* species are classified among moderately salt-tolerant forage legume (Munns and Tester,

2008; Al-Farsi et al., 2020; Mbarki, 2020). These glycophyte plants have possibility to grow under salty condition and were competent to improve the quality and quantity of pasture (Moulai and Fyad Lameche, 2014; 2017, Bakhti and Fyad Lameche, 2021). The selection of annual tolerant species can constitute an important alternative to reduce significantly improvement programs. In flowering plants, selection that acts on the gametophyte stage (pollen grains and ova) can influence the sporophyte stage (seeds and plants). If the same genes are expressed in both stages, then selection of genes at the gametophyte stage can lead to the evolution of traits genetically corresponding to the sporophytic stage. This correspondence between the genes (code for protein and enzyme) which are expressed at the level of the sporophyte and the gametophyte makes it possible to ask some questions such as Are specific gene sets transcribed during male gametophyte development? Or is there an overlap in the sporophyte and gametophyte genetic programs?

The overlap of gene expression between the two stages is previously known as "overlapping" (Mulcahy, 1974). The "fitness" of the male gametophyte is clearly transferred to the resulting sporophyte because over 60% of the genes expressed in the sporophyte phase of the life cycle are also expressed in the male gametophyte (Tanksley et al., 1981; Willing and Mascarenhas, 1984; Mulcahy and Mulcahy,

1987). There are two categories of overlapping: (1) A Structural "overlapping", which reflects the importance of the overlap of the pollen and sporophytic genetic domains without prejudging their function. (2) A functional (adaptive) "overlapping". This concept establishes a correspondence between the vigor of the gametophyte and that of the sporophyte. While expression studies offer valuable evidence for the anticipation of genes expressed in the gametophyte and display notable convergence with the sporophyte, the relationship between this gene expression and its impact on sporophyte fitness is not always evident (Beaudry et al., 2020). To decipher this overlap model, a selective pressure was applied to three annual species of the *Medicago* genus. This work focused on two parts, a first devoted to biometric measurements and the second related to biochemical approach by the study of the esterase activity.

Results

Expression of overlap between seed and pollen germination rate

The sporophyte and gametophyte data analysis showed that the seven ecotypes differed significantly between themselves with or without stress application for the germination rate parameter (Fig.1). However, concentrations less than or equal to 68 mM of NaCl only cause a slight decrease. The 102 mM concentration mostly causes an average decrease, allowing discrimination between the studied species with respect to tolerance to salinity. But a strong reduction in the germination rate was recorded at the 137 mM concentration. The germination rates recorded in the control are always higher than the rates recorded under stress with one exception, whereas in the ecotype A4 the value of the control is lower than that of the treated concentration in both sporophyte and gametophyte phases (Fig.1).

At control T0, ecotype Tr1 has the highest germination rate 0.94 ± 0.092 compared to other ecotypes in the sporophyte phase. It keeps the same germinal power with the value 0.77 ± 0.177 in the gametophyte phase. It explains that the same germinal power is found in the two phases of development. In addition, the decrease in germination rate under stress conditions may explain the same hypothesis for overlap tolerance. In general, the germination rate is being little affected at 68 mM concentration of NaCl in both phases for all groups, whereas from high concentrations, the germination rate of the majority of ecotypes is strongly affected (Fig.1).

The ecotype effect is highly significant for all treatments, as revealed by analysis of variance ANOVA tests at 1% (Table 1) with one exception for T3 in sporophyte phase which is not significant. This highest concentration could prevent all seed groups to germinate. However, the value of the highest F statistic is noted under a concentration of 102 mM; indicating that this concentration is the best screen to determine the tolerant and sensitive genotypes towards a salt stress among all analyzed groups.

Expression of overlap between seedlings and pollen tubes length

Figure 2 represents the morphological aspect of 9-day-old seedling of ecotype A4 for and the microscopic aspect of pollen tubes after 2 hours of germination. In ecotype A4, the seedling and the pollen tube growth are extremely longer

than T1, T2 and T3 treatments, resulting in earlier germination (Fig.3). Irrespective of the ecotypes, increasing salinity significantly delayed seedling establishment and inhibited pollen tube growth. Finally, the seedlings and pollen tubes growth were decreasing, but both inter- and intra-specific differences were observed, whereas ecotype Tr1 exhibited better performance than the rest of ecotypes. This was evident at 102 mM NaCl, in which the highest value was recorded in Tr1 (1.88 ± 1.969 cm), followed by C2 (1.26 ± 1.674 cm), A3 (1.06 ± 0.380 cm), whereas rest of ecotypes have shown the lowest value (Fig.3).

Overall, the ANOVA analysis (Table2) showed also a highly significant effect of ecotype on the growth in terms of seedling (sporophyte) and pollen tube length (gametophyte).

Tolerance index and expression of overlap for the vigor

The calculated values of the tolerance index IT2 by ecotype and by stage are presented in Table 3. They are indicating that after a treatment (T2), the growth of pollen tube is considerably decreased but the classification of ecotypes remains the same for both stages of development with the exception of ecotypes C1 and Tr1 which are slightly changed at different stages. The classification of the different ecotypes according to their tolerance to NaCl is shown in Table 3. At the 68 mM concentration, the A4 ecotype records the highest tolerance index of 0.63 and 0.35 in sporophyte and gametophyte, respectively. The rest of the populations have values between 0.64 and 0.33 in sporophyte stage. However, the values ranged between 0.35 and 0.03 in gametophyte stage IT2 (Table 3).

In this study, seedlings and pollens survived vigorously after exposed to salt stress. The tolerant A4 and sensitive C1 and Tr1 ecotypes are the same in sporophyte and gametophyte stages (Table 3). These results are explained by the presence of gameto-sporophyte overlap for the fitness or vigor character. While we are unable to distinguish between strict salt tolerance on the part of the sporophyte and on the part of the male gametophyte, we did detect a possible overlap between gametophytic and sporophytic responses against the salinity. The classification of ecotypes according to the IT2 tolerance index is the same for the two developmental stages, under the T2 concentration. We propose that the mechanisms controlling the stress response in the both sporophyte and gametophyte phases would be the same.

Overlap in esterase isoenzymes activity and expression

In order to find biochemical correlations between the two phases, the results of the esterase electrophoresis were also compared. For this, we compared the electrophoresis profiles at the diploid (2N) stage of the same C1 ecotype of *M. ciliaris* with those at the haploid (N) stage (Figure 3). There are four bands of esterase (Est-1, Est-2, Est-3 and Est-4) migrate to almost identical positions, suggesting that they might have a common genetic origin. However, they need a straightforward genetic analysis (Fig.4). The results of this study show that the esterase isoenzymes activities are present in pollen and are encoded by the same genes in the sporophyte as in the male gametophyte. Moreover, this correspondence of genes expression explains per the presence of four band patterns in the both phases. But the variation in band intensity is very important in sporophyte than male gametophyte. This intensity correlates with the change of NaCl concentration levels (Fig.4).

Discussion

The results suggest that seed germination decreases under the application of salt stress, which is generally characteristic of glycophytes (Edwéard Glenn et al., 1999; Mann et al., 2020). While differences between genotypes at three concentrations are clear, the high saline concentration also reduces pollen germination. This reduction is significant for several genotypes.

In general, NaCl stress had an effect on pollen performance and seed vigor, by both reductions on pollen germination rate and seed germination rate. Furthermore, an interaction between the effect of NaCl stress and genotype has been recorded in both sporophyte and gametophyte stages. This may explain the evidence of overlap between two developmental phases for germination parameter. These results are corroborating with those found by Ammouri and Fyad-lameche, (2012).

In addition, values recorded for the salt treated seedlings and pollen were markedly lower than in control, reflecting the adverse effect of salinity on the growth process in two phases. Yet, this impact was much more pronounced in gametophyte as compared to sporophyte. Debez et al. (2020), demonstrate that, the increasing salinity had a significant harmful effect on the root length of 14-day-old barley seedlings. Salt stress is most significant during seed germination and early growth of lucerne, reducing the germination rate, relative growth rate and radicle elongation (Li et al., 2010)

Many years ago, Strogonov (1974) reported that salt stress reduced root growth in lucerne by 89%. Many studies demonstrated that salt stress can also affect seed germination and inhibit elongation of seedlings (Greenway and Munns, 1980; Bohnert et al., 1995; Dash and Panda, 2001; Sairam and Tyagi, 2004). In general, the salt stress inhibits or delays seed germination and shoot and root growth. Our results show that salt stress alters seed vigor and inhibits radicle growth. Also in gametophyte, this effect was more pronounced in sensitive ecotypes. In other sporophyte study, the same reduction of seedling length was also found for all populations of *M. ciliaris* species at 200 mMNaCl (Mbarki et al., 2020).

A comparative study of the salinity tolerance of the male gametophyte and the sporophyte has been undertaken on six ecotypes of annual species of *Medicago* by Amouri and Fyad-lameche (2012). Our study leads to the same results.

Globally, there is a genotypically different response of *Medicago* species to saline stress. Plant reactions to saline stress are the result of the complex interplay between different morphological, physiological and biochemical processes (Semchuk, 2012; Nawaz et al., 2020). However, the tissue-specific signal transduction in response to the high salinity remains unexplored in seed tissues (Chen et al., 2021).

In plants, the vigorous selection of pollen has been shown to not only affect fertilization success rates but also directly improve offspring fitness (Simone, 2019). Lucerne (*Medicago sativa* L.) selected for vigor in a non-saline environment maintained growth under salt stress (Kapulnik et al., 1989). Staining of esterase zymograms showed four bands (Est1, Est 2, Est 3 and Est4). They were found in the both sporophyte and the gametophyte phases irrespective of the

presence or absence of NaCl stress. In both phases the esterase activities (band Est-4) increased with increasing of salinity levels (Fig.4). However, the changes in band intensity in seedling were higher than germinated pollen treatments. In other case study, esterase activities in shoots increased under low saline treatments and decreased in response to increased saline treatments (Radić and Pevalek-Kozlina, 2010).

According (Munns and Tester, 2008), high concentrations of sodium ions can inhibit activity of many essential enzymes in the two phases of development.

On the contrary, as observed by Siegel et al. (2018), even though there is an extensive overlap of approximately 90% in genetic identity between the sporophyte and gametophyte stages of *P. amorphum*, each phase exhibits a unique transcriptional profile. The conclusive evidence that transcription and translation of the haploid genome occur during pollen development was obtained from studies of several dimetric enzymes (Mascarenhas, 1989). Calculation of gameto-sporophytic genetic overlapping rate based on enzyme resulted in 100% for *pearl millet* (on 12 isozymes) and 84% for maize (on 19 isozymes) (Le Thi et al., 1992). Appearance of four-bands for esterase multimeric enzymes in seed and pollen clearly show transcription of the corresponding genes. Therefore, this gameto-sporophyte genetic overlapping is not due to an accumulation of proteins prior to meiosis but is explained by an early synthesis of transcriptions at the haploid level. The appearance of these esterases genes in pollen clearly shows that the process of their synthesis happens immediately after the maturation of the microspores.

The strong gameto-sporophyte genetic overlap for pearl millet supports this assumption that selection forces acting at the haploid level during pollen competition can be very important in the evolution of *pearl millets* and, more generally, of angiosperms (Le Thi et al., 1992). It appears that, for many vascular plants, approximately 60% of the genes expressed in the sporophyte are also expressed during the gametophyte stage. For genes that are equally expressed in the male gametophytic and in the sporophytic generations, it is to be expected that the efficiency of gametophyte selection is much greater than selection applied to the sporophyte, in consequence of the population size and the haploid state. The male gametophytic population is much larger than the sporophytic population (Le Thi et al., 1992). Gametophytic selection as a tool in plant breeding refers to methodologies based on the application of selection pressures to the male gametophytic generation with the purpose of improving traits of the sporophytic progeny. Some authors use selection at this stage to increase the feasibility of pollination and transmit genes of agronomic interest to the next sporophytic generation (Alisher et al., 1995). It is for the reason that gametophytic selection for salt stress tolerance (Vasiliy, 2000) or other abiotic stress has been widely studied in different plant species. It is considered a tool for improving stress tolerance (Baloch, 2007). However, selection of gametophyte can only affect the fitness of the sporophyte if there is a significant overlap between the genes expressed in the gametophyte and sporophyte generations (David and Twell, 2003).

Table 1. ANOVA for the germination rate character in the sporophyte (2N) and gametophyte (N) for control and in stress (T1, T2 and T3).

Phases	Concentrations	ddl	MC	F	p
Sporophyte (2N)	T0	6	0.575375	9.956128	0.000000 ^{***}
	T1	6	0.452526	3.844133	0.003211 ^{***}
	T2	6	0.544684	2.924204	0.01616 ^{**}
	T3	6	0.175018	0.811029	0.566468 ^{ns}
Gametophyte (N)	T0	6	1.628351	24.97753	0.000000 ^{***}
	T1	6	2.311155	25.20014	0.000000 ^{***}
	T2	6	0.949935	9.278134	0.000000 ^{***}
	T3	6	1.394184	19.94224	0.000000 ^{***}

ns: not significant; **: significant at 0.05 ; ***: highly significant at 0.01; df : degree of freedom; MC: Mean square; F: Fisher coefficient; p: Probability;

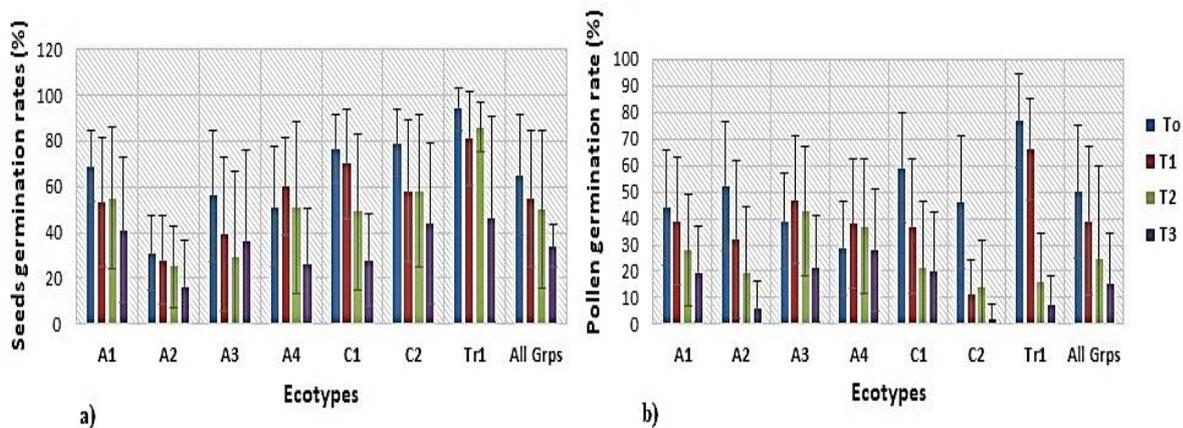


Fig 1. Germination rate average with SD (bars) for individually ecotype (A1: genotype *M.acl1*; A2: genotype *M.acl2*; A3: genotype *M.acl3*; A4: genotype *M.acl4*; C1: genotype *M.cil1*; C2: genotype *M.cil2* and Tr1: genotype *M. tru1*) and for grouped ecotypes (All Grps) at different levels of NaCl concentrations; T0= 0mM; T1=68mM; T2=102mM and T3= 137mM. **a)** Seed germination rate \pm SD (%) of sporophyte phase diploid (2n) **b)** Pollen germination rate \pm SD (%) of gametophyte phase haploid (n).

Table 2. Test of the ecotype effect by ANOVA for the average length under control and stress conditions (T1, T2 and T3) in both sporophyte and gametophyte phases.

Phases	Traitements	df	MC	F	p
Sporophyte (2N)	T0	6	185.5522	13.85973	0.000000 ^{***}
	T1	6	60.51312	8.48250	0.000005 ^{***}
	T2	6	16.89843	3.16289	0.011899 ^{**}
	T3	6	4.19635	1.351895	0.256305 ^{ns}
Gametophyte (N)	T0	6	662164.6	135.3622	0.000000 ^{***}
	T1	6	119998.3	115.6604	0.000000 ^{***}
	T2	6	42964.68	92.54303	0.000000 ^{***}
	T3	6	12490.38	136.3854	0.000000 ^{***}

ns: not significant; **: significant at 0.05 ; ***: highly significant at 0.01; df : degree of freedom; MC: Mean square; F: Fisher coefficient; p: Probability.



Fig 2. Morphological appearance of seedlings and pollen tube for tolerant ecotype A4, cultured in different levels of NaCl concentration T0= 0mM; T1=68mM; T2=102mM and T3= 137mM. **a)** Seedling sporophyte (2n diploid) with scale cm; during 9 days growth period. **b)** Pollen tube male gametophyte (n haploid) with scale μ m; during a 1 hour growth period.

Table 3. Classification of ecotypes according to tolerance index IT2 for the average length of seedlings and pollen tubes length.

Sporophyte (2N)		Gametophyte (N)		Ecotypes
Ecotypes	IT2	Ecotypes	IT2	Classification
A4	0.63	A4	0.35	{1}
A3	0.51	A3	0.28	{2}
A1	0.47	A1	0.19	{3}
C2	0.47	C2	0.14	{4}
A2	0.45	A2	0.12	{5}
Tr1	0.33	C1	0.09	{6}
C1	0.29	Tr1	0.03	{7}

A1: genotype *M.acl1*; A2: genotype *M.acl2*; A3: genotype *M.acl3*; A4: genotype *M.acl4*; C1: genotype *M.cil1*; C2: genotype *M.cil2* and Tr1: genotype *M. tru1*.

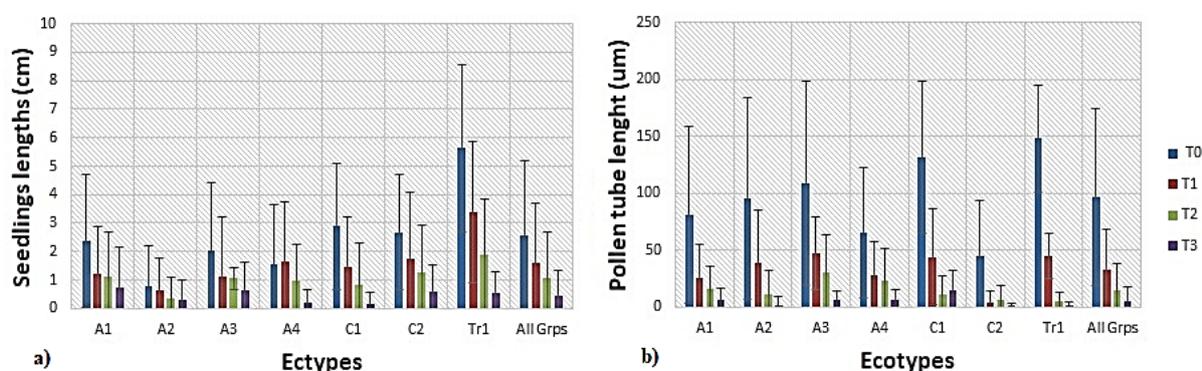


Fig 3. Lengths average with SD (standard deviation bars) for seedling and pollen tube for individually ecotype (A1: genotype *M.acl1*; A2: genotype *M.acl2*; A3: genotype *M.acl3*; A4: genotype *M.acl4*; C1: genotype *M.cil1*; C2: genotype *M.cil2* and Tr1: genotype *M. tru1*) and for grouped ecotypes (All Grps) at different levels of NaCl concentrations; T0= 0mM; T1=68mM; T2=102mM and T3= 137mM. a) Seedlings lengths average \pm SD (cm) sporophyte phase; diploid (2n). b) Pollen tube lengths average \pm SD (μ m) gametophyte phase; haploid (n).

Table 4. List of utilized species and ecotypes of *Medicago genus*; their origin and altitude based on laboratory data.

Species	Genotype of ecotypes	Experimental code	Origin	Altitude (m)
<i>M.aculata</i>	<i>M.acl1</i>	A1	Syria	80 m
	<i>M.acl2</i>	A2	Algeria	800 m
	<i>M.acl3</i>	A3	Algeria	710 m
	<i>M.acl4</i>	A4	Algeria	500 m
<i>M.ciliaris</i>	<i>M.cil1</i>	C1	Algeria	470m
	<i>M.cil2</i>	C2	Algeria	470m
<i>M.truncatula</i>	<i>M.tru1</i>	Tr1	Algeria	-

- Old collection of laboratory

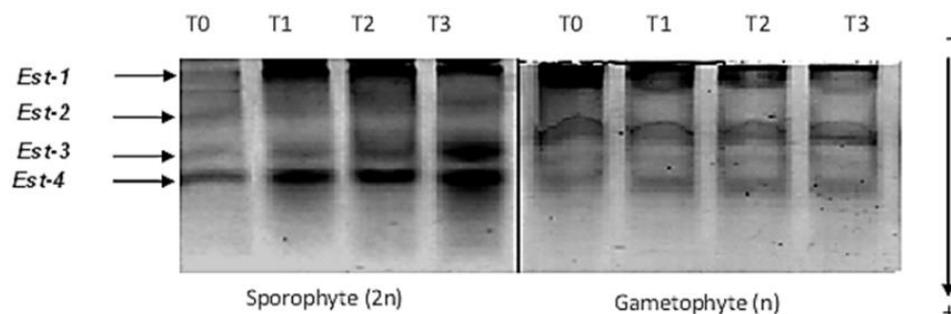


Fig 4. Electrophoretic patterns comparison of seedlings (2n) and pollen tube (n) esterase isoenzymes activity for the same ecotype C1, under control (T0= 0mM) and NaCl stress conditions (T1=68mM; T2=102mM and T3= 137mM) during 9 days and 1 hour growth period respectively. Gels were incubated in a solution of both substrates together. Equal volume per well of sample was loaded onto each gel lane. *Est1 Est2, Est3 and Est4* arrows indicate the number of obtained esterase bands.

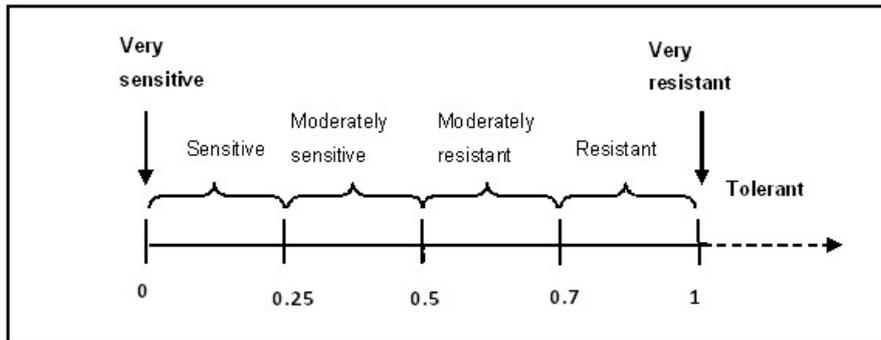


Fig 5. Scale proposed for the classification of ecotypes according to their tolerance index (TI).

Materials and Methods

Plant material

The experiments were carried out in a greenhouse at Oran 1 Ahmed Ben balla University; Laboratory of Biotechnology of Rhizobia and Plant Breeding Algeria, using seeds (*Medicago* species) were obtained from the same laboratory. The seeds of three annual *Medicago* species including *M. aculeata* (with 4 ecotypes A1, A2, A3 and A4), *M. truncatula* (one ecotype Tr1) and *M. ciliaris* (with 2 contrasting ecotypes C1 and C2 to salinity stress) (Table4).

Plant culture and salt treatments

For each species, intact seeds, which were homogeneous and identical in size and colour and free from wrinkles, were chosen then were sterilized by %5 sodium hypochlorite and washed three times by distilled water. Salinity stresses with different NaCl concentrations T0=0mM; T1=68mM; T2=102mM and T3= 137mM) were applied according to Ammouri and Fyad-lameche (2012). Ten scarified seeds for each treatment were placed on the wet filter paper in sterile petri dish with 91 mm of diameter and kept in a germinator under dark conditions at 21 ± 2 °C for three days, when two leaves were developed. After three days of germination, uniform seedling of each genotype was transferred into others petri containing sterile sand and Knop's solution. The seeds were left to grow inside the culture chamber under natural lighting, $(25/19) \pm 2$ °C (day/night) and 70% relative humidity. At six days from transplanting and a total of 9 days of germination, the seedlings of 4 treatments were harvested, and the following data were recorded: germination rate and the length of young seedlings (to realize the sporophyte study). According to the conditions of ISTA (2015), the seed is considered as germinated if the length of the root is equal to or greater than 1 millimeter. The total lengths are measured on the 10 plants by ecotype and by treatment. Four readings (T0, T1, T2 and T3) per seedling and per ecotype of the germination rate and the length of young seedlings are taken on the ninth day. The experiment was carried out based on randomized complete design with eight replications. In each repetition, each ecotype is represented by ten individuals.

Collection of pollen and germination medium

The plant material is the same as that used in the analysis of the sporophyte phase. Pollen is collected from plants grown in the greenhouse under natural light supplemented with fluorescent light to achieve a 16-h photoperiod or from field

grown plants. Pollen was collected in the morning and used the same day. However, we have stored the pollen without serious decreases in pollen viability. Five plants for each ecotype were sampled for flower buds at the required stage with mature pollen grains. From each plant 3 to 6 flowers were taken before fertilization, before their outbreak or before the flowers opened, exactly at the stage where the (Sepals / petals) ratio is 0.7.

The pollen from these flowers is mixed, and then applied evenly with a fine brush on cellophane paper. The latter is placed on the glass slide, on a drop of germination medium. The germination medium consists of 100 ppm of boric acid, 100 ppm of KNO₃, 300 ppm of Ca (NO₃)₂ and 10% of sucrose, with pH adjusted to 6.8(de Vienne, 1979). The T0 medium represents the control with 0 mM NaCl. The T1, T2 and T3 media differ in their NaCl concentration with 68, 102 and 137 mM, respectively.

One hour after germination of the pollen grains in dark at 23°C, two parameters (germination rate and the length of the pollen tube) were measured. A pollen grain is considered germinated when the length of its tube is greater than the diameter of the pollen grain. The length of the pollen tube is expressed in micrometric units (µm). The count consists of counting the pollen grains germinated on 10 fields per slide, and 10 lengths of pollen tubes are measured per field. Observations are made under an optical microscope, objective x10.

Extraction of esterase isoenzymes

The extraction is carried out from young seedlings aged 9 days for the T0 control on the three treated T1, T2, and T3. The sporophytic esterase extraction is carried out cold in a mortar in the presence of sand and Tris-KCl buffer, pH = 7. The pollen of a one hundred flowers is germinated at 25 °C, after one hour the contents of the 4 slides (T0, T1, T2 and T3) are collected using a razor blade. The gametophytic esterase extraction is carried out in a cold ceramic plate in the presence of sand and the same buffer used in sporophyte phase. The both extracts are then subjected to cold centrifugation (4 °C.) for 15 to 20 minutes at a speed of 13,000 rpm. All extracts are stored in a cryopreservator containing liquid nitrogen until the day of electrophoresis.

Electrophoresis analysis

The migration is carried out either on discontinuous polyacrylamide gels containing a gel with a concentration of 4% and a separation gel at 8% with a thickness of 0.5 mm, or on continuous 8% acrylamide gels, in Tris-Borate-EDTA buffer at pH = 8.3, with NP 10, TEMED and ammonium persulfate(Fyad-Lamèche, 1998 modified). The tank buffer

consists of gel buffer diluted 10 times. The migration takes place at 4 °C for 6 h under constant amperage of 30 mA and a voltage of 150 V. It is controlled by the displacement of the bromophenol blue. The development takes place in a solution containing 0.1 M Na₂HPO₄ at pH = 6.2, after adding a 1% α naphthyl butyrate substrate mixed with acetone and Fast Blue RR salt. Half an hour after the appearance of the bands, the gels are fixed in a 7% acetic acid solution and then stored in 7% glycerol. After visualization, the gels are dried and stored for the reading.

Statistical analysis

Statistical tests selected for the analysis of germination and seedling growth traits at the gametophytic phase are the same as those used for sporophytic analysis. The germination percentage data were transformed using the root mean square (sqrt) and arcsine (asin) functions in the TANAGRA statistical package (2005). After, the usual transformations to normalize the variables, all data were analyzed using the standard analysis of variance and means ±SD were compared with Student-Newman Keul's test as described by Steel and Torrie (1981). For both phases, the results were analyzed by comparing (F) values obtained from a one-way ANOVA using the same statistical package and Microsoft Excel 2010 with (at $p \leq 0.05$) as significance level.

Calculation of tolerance index

To determine the relative tolerance of the different ecotypes to salt stress, a Tolerance Index (TI) was calculated. It is equal to the ratio between the mean value noted under stress on that of the control (for example: $TI_1 = T_1 / T_0$). In this way, it is possible to compare the ecotypes among themselves by calculating the three TIs. This index is between 0 and 1. It was considered that tolerant plants have a higher tolerance index than sensitive plants. A classification scale for the tolerance of ecotypes to salt stress has been proposed (Fig.5).

Conclusion

The improvement of salt stress tolerance has long been a goal in crop breeding. However, it is not easy to accomplish this goal because plant tolerance to high salt level is the result of a variety of mechanisms acting at both the sporophytic and the gametophytic levels. This study concerning salinity tolerance in annual species of the *Medicago* genus made it possible to select the most tolerant ecotype by comparing the germination rate as well as the seedlings and the pollen tubes length. Also, the electrophoresis of the esterase activities revealed more information. The existence of overlapping genetic expression between the two development stages makes gametophyte selection an efficient, rapid and economical technique. It can be applied to the selection of genotypes tolerant to abiotic stress in *Medicago* species and in other legumes of selection program for their introduction into arid and semi-arid areas.

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